

Molecular analysis of avian H7 influenza viruses circulating in Eurasia in 1999–2005: detection of multiple reassortant virus genotypes

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are EU158100–EU158178.

Supplementary material is available at the end of this paper.

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Avian influenza infections by high and low pathogenicity H7 influenza viruses have caused several outbreaks in European poultry in recent years, also resulting in human infections. Although in some cases the source of H7 strains from domestic poultry was shown to be the viruses circulating in the wild bird reservoir, a thorough characterization of the entire genome of H7 viruses from both wild and domestic Eurasian birds, and their evolutionary relationships, has not been conducted. In our study, we have analysed low pathogenicity H7 influenza strains isolated from wild and domestic ducks in Italy and southern China and compared them with those from reared terrestrial poultry such as chicken and turkey. Phylogenetic analysis demonstrated that the H7 haemagglutinin genes were all closely related to each other, whereas the remaining genes could be divided into two or more phylogenetic groups. Almost each year a different H7 reassortant viruses was identified and in at least two different years more than one H7 genotype co-circulated. A recent precursor in wild waterfowl was identified for most of the gene segments of terrestrial poultry viruses. Our data suggest that reassortment allows avian influenza viruses, in their natural reservoir, to increase their genetic diversity. In turn this might help avian influenza viruses colonize a wider range of hosts, including domestic poultry.

INTRODUCTION

Influenza A viruses are negative-sense RNA viruses with a genome divided into eight segments coding for 11 proteins (Hay, 1998; Chen *et al.*, 2001). They are further classified into subtypes according to the two surface glycoproteins: 16 different haemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified so far (Fouchier *et al.*, 2005). Influenza A virus host range includes wild waterfowl (their natural reservoir) and several other avian and mammalian species, including man (reviewed by Webster *et al.*, 1992). Ecological studies have established that all influenza A viruses in nature are derived from strains circulating in wild aquatic birds, where they appear to be usually apathogenic (Webster *et al.*, 1992). Transmission of avian influenza viruses (AIVs) from wild birds can cause disease in domestic poultry, and transfer of gene segments from avian to human viruses by reassortment has resulted in at least two human pandemics (Alexander, 2000; Scholtissek *et al.*, 1978). Of the 16 HA subtypes, two (H5 and H7) have the potential to become highly pathogenic (HP) for domestic poultry (Wood *et al.*, 1993; Senne *et al.*, 1996). Moreover, since the 1997 H5N1 influenza outbreak in Hong Kong, influenza A viruses belonging to several avian subtypes, including H5 and H7 HP viruses, have shown their ability to infect humans directly, suggesting that preliminary adaptation in an intermediate host through the generation of human–avian virus reassortants is not an absolute requirement to infect humans (Claas *et al.*, 1998; Fouchier *et al.*, 2004; Lin *et al.*, 2000; WHO, 2005). At present, the continued circulation of HP H5N1 viruses in domestic birds in Eurasian and northern African countries, accompanied by a growing number of human infections, mostly fatal, increases the likelihood that this virus may become transmissible from human to human and result in a pandemic (WHO, 2005). However, the type of aquatic bird, infected with influenza viruses, capable of transmitting to domestic poultry is still unclear. In one instance, a H7N3 virus from wild waterfowl was shown to be the direct precursor of viruses of the same subtype that caused widespread and prolonged outbreaks of low pathogenicity (LP) avian influenza (AI) among commercial poultry farms in northern Italy in 2002–2003 (Capua *et al.*, 2002), during which serological evidence of H7 virus transmission to poultry workers was obtained (Puzelli *et al.*, 2005). Genetic analyses showed that only a few nucleotide and amino acid changes throughout the genome differentiated the wild from the domestic avian virus (Campitelli *et al.*, 2004). Given the important role of wild bird viruses for the emergence of viruses pathogenic for both domestic poultry and humans, and the fact that measures to contain these viruses are impossible to implement in the wild, improved knowledge about the ecology of influenza viruses and the properties of their gene pool in feral waterfowl is crucial to develop interventions aimed at limiting the risk of transmission to other species. Recent

reports have highlighted the existence of multiple sublineages within the major lineage of AIVs isolated from their natural reservoir in North America, and suggested that reassortment events in the wild waterfowl reservoir occur more frequently than previously thought (Hatchette *et al.*, 2004; Widjaja *et al.*, 2004). However, little data are available on the genetic heterogeneity and extent of gene pool mixing among wild and domestic bird viruses isolated in the entire Eurasian region, despite their importance for animal and human health. Therefore, we have analysed and compared the genome of a group of H7 subtype AIVs isolated from Italy over a 6 year period with those co-circulating in Europe and China at the same time in order to characterize the genetic heterogeneity among H7 subtype viruses isolated from birds, including wild and domestic species, between 1999 and 2005. We also evaluated the extent of reassortment in this gene pool and its role in the generation of viruses capable of infecting domestic avian species, and analysed the molecular determinants relative to receptor binding, host adaptation, virulence and antiviral susceptibility.

METHODS

Sample collection and virus isolation.

Cloacal samples were obtained from wild mallard ducks (*Anas platyrhynchos*) in central and northern Italy, from turkeys and chickens raised in commercial poultry farms in northern Italy, and from domestic ducks raised in farms at Poyang Lake, China. Poyang Lake is located in the north Jiangxi Province, is the largest freshwater lake in China and is a major overwintering site for migratory birds in eastern Asia (Li & Mundkur, 2004). For influenza A virus detection and isolation from wild and domestic waterfowl from Italy, cloacal swabs were processed as follows: pools of five to six faecal specimens were prepared, viral RNA was extracted using the RNeasy kit (Qiagen), and RT-PCR with primers M52C and M253R, which are specific for a conserved region of the influenza matrix protein, was performed as described previously (Fouchier *et al.*, 2000). Samples from PCR-positive pools were inoculated into 10-day-old embryonated specific-pathogen-free hen's eggs, and influenza isolates were identified by both the haemagglutination test (according to standard procedures) and a double-antibody 'sandwich' ELISA for the detection of influenza A virus nucleoprotein (Siebinga & de Boer, 1988). For virus isolation from turkey and chicken, cloacal and/or tracheal samples were inoculated into embryonated hen's eggs, as described above. For those viruses isolated from China,

